Bio-validation of Steam Sterilization

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INTRODUCTION

The science that underpins steam sterilization is well known and has been long established. It is the preferred method of sterilization in the pharmaceutical industry; it is used for sterilization of aqueous products in a wide variety of presentations, for sterilization of equipment and porous materials required in aseptic manufacture, in microbiology laboratories for sterilizing media and other materials, and for sterilization of "massive" systems of vessels and pipework [steam-in-place (SIP) systems]. Numerous rules and guidelines have been published on the topic, yet steam sterilization and particularly bio-validation of steam sterilization is still a subject for controversy and debate.

The purpose of this article is to reexamine the bio-validation of steam sterilization, to clarify what is needed and why it is needed, and to distinguish the scientific need from the regulatory need in areas where they may appear to differ.

PRINCIPLES

Micro-organisms are inactivated when metabolically irreversible deleterious intracellular reactions occur. At high temperatures and in the presence of moisture, as in steam sterilization, the energy input from the steam inactivates micro-organisms by denaturation of intracellular proteins.

Although these reactions are complex at a biochemical level, their kinetics approximate to reactions of the first order. Thus, the kinetics of inactivation of populations of pure cultures of micro-organisms take the typical exponential form of reactions of the first order. What this means in experimental practice is that there is a linear relationship[1] when numbers of micro-organisms held at high temperatures are plotted on a logarithmic scale against time plotted on an arithmetic scale (Fig. 1).

There are two highly significant points to be drawn from the kinetics of inactivation of micro-organisms.

First, logarithmic scales never reach zero. This means that there can never be any specifications for temperature and time which can guarantee that all micro-organisms contaminating items are going to be inactivated. However, the consequences to patients of micro-organisms surviving in allegedly sterile pharmaceutical preparations can easily be fatal. Thus, sterilization processes must be specified to ensure that the probability of micro-organisms surviving in treated items is low enough to ensure patient safety. The accepted low probability indicated in the pharmacopeias is that there should be not more than one chance in one million of viable micro-organisms surviving on a treated item. This is called a probability of nonsterility of 10^{-6} or a sterility assurance level (SAL) of 10^{-6}.

Second, the inactivation curve takes a regular form. This means that steam sterilization is a predictable process as long as some information is available (or can be safely assumed) about the numbers and thermal resistances (D_T-values, Fig. 1) of the micro-organisms contaminating items before treatment. This is important because there is no practical way to test for the achievement of SALs of 10^{-6}. The sterility or nonsterility of items cannot sensibly be confirmed in a treated item except by sacrificing the item. The pharmacopeial *test for sterility* is a sacrificial test with statistical limitations which have been so extensively criticized[2–6] over so many decades that they should now be well understood. For instance, the sample of 20 items which is generally required in the test would allow a batch containing nonsterile items at a frequency of 1:100 to be passed on four out of every five occasions. This falls a long way short of being able to detect deviations from a standard of not more than one nonsterile item in one million.

Justification of the reliable achievement of SALs of 10^{-6} for particular pharmaceutical items treated according to particular specifications of temperature and time in particular sterilizers is predicated on the regularity and predictability of steam sterilization processes. The means of justification are through scientifically based development of sterilization specifications and sterilizer parameters,

Encyclopedia of Pharmaceutical Technology
DOI: 10.1081/E-EPT120003776
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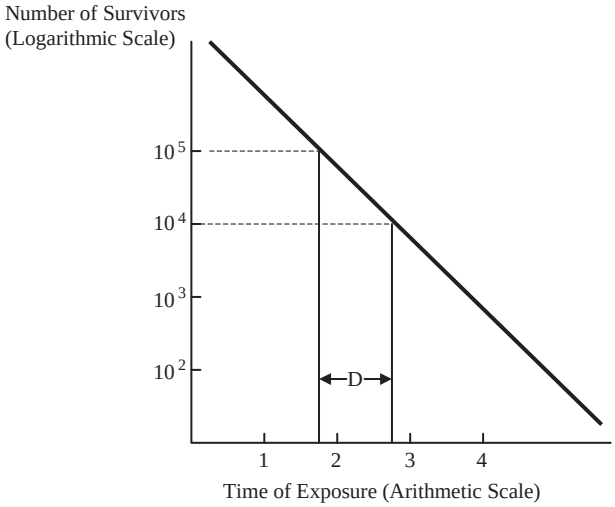

Fig. 1 Exponential inactivation of micro-organisms (the survival curve).

and through subsequent validation of the specified processes.

DEVELOPMENT OF STERILIZATION SPECIFICATIONS AND STERILIZER PARAMETERS

The development of sterilization specifications differs from the development of sterilizer parameters. Both differ from validation (Fig. 2).

Pharmaceutical Products and Materials for Aseptic Manufacture—Sterilization Specifications

For pharmaceutical products and materials used in connection with aseptic manufacture, sterilization specifications apply to conditions of temperature and time, or F_0, or combinations of F_0, temperature and time to which the contaminating micro-organisms themselves must be exposed over the "hold" period of the sterilization process. In practice, this means actually within aqueous products, on the surfaces of rubber stoppers or metal machine parts, or within the folds of cartridge filters, etc.

The sterilizer parameters are the practical criteria that must be specified to ensure that the sterilization specification is delivered to all parts of the load. They always include specifications for temperature and time, but it is important to recognize the distinction between sterilizer parameters applying to the machine settings on the autoclave console, and sterilization specifications applying to actual conditions within the load. Essential sterilizer parameters also include other specifications, e.g., for load configuration, number and depth of prevacuums, cooling characteristics, etc.

Sterilization specifications are product specific. Sterilizer parameters are specific to combinations of product, presentation, and autoclave.

Sterilization specifications may be determined from theoretical considerations or from laboratory data and are within reason transferable from presentation to

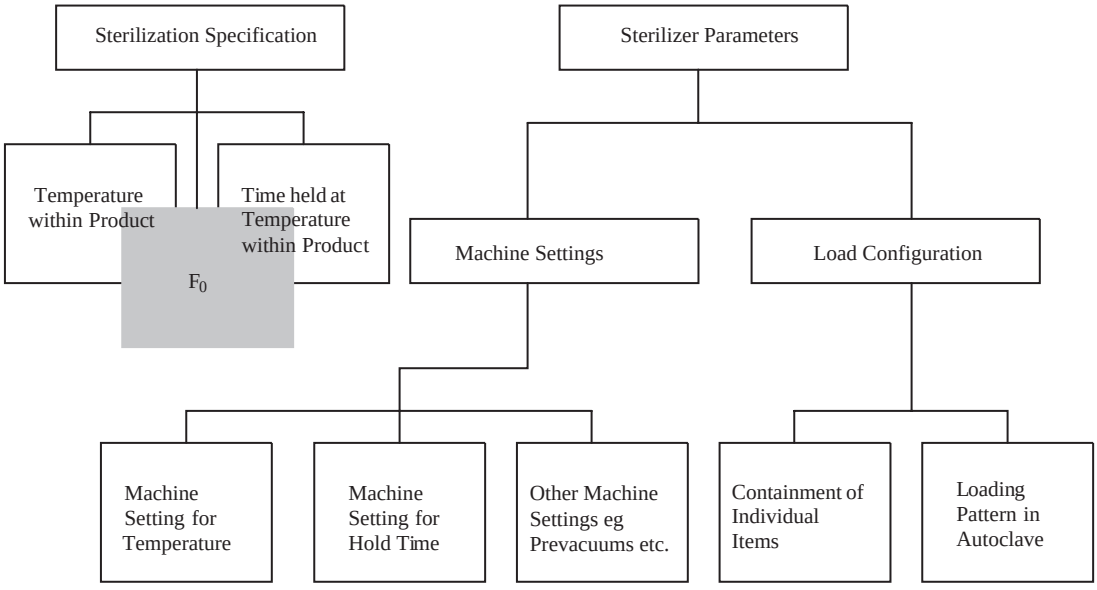

Fig. 2 Sterilization specifications and sterilizer parameters.

presentation, e.g., from 1 ml ampules to 5 ml vials to 50 ml bags. The sterilizer parameters required to deliver the sterilization specification to these presentations differ within the same autoclave and from one autoclave to another according to differences in load configurations, chamber size, steam entry points, control systems, etc. Sterilizer parameters are not transferable and must be developed empirically for each autoclave.

Sterilization specifications should be easy to develop. The pharmacopeias allow sterilization specifications to be developed from a basis of no actual data concerning the numbers and thermal resistances of micro-organisms actually contaminating the items to be sterilized. Although this statement may appear initially to be barren of scientific reason, this is in fact not the case. What the pharmacopeias provide are either a recommended overkill specification (*PhEur*) or principles for specification development (*USP*) that incorporate amounts of thermal lethality well in excess of that which could ever be practically required to obtain SALs of 10^{-6}—for this reason they are called "overkill" specifications.

In the *European Pharmacopoeia* (*PhEur*), a specification of 121°C for 15 min is given as the reference condition for overkill sterilization of aqueous preparations. The *United States Pharmacopeia* (*USP*) defines a lethality input of 12*D*. These specifications merit some examination in detail.

It is worth considering the thermal resistances of micro-organisms found in pharmaceutical manufacturing environments. It is extremely rare for anyone to have isolated thermally resistant bacteria with D_{121}-values (in water) of greater than 0.3 min. The author of this paper has experience of having determined a D_{121}-value (in water) of 0.8 min for an environmental isolate of *Bacillus coagulans*, but this was several decades ago and was done with what would now be considered fairly primitive equipment. It is therefore probably quite reasonable to assume a worst case D_{121}-value of 1 min. Given this "worst case," the *PhEur* overkill specification of 121°C would deliver 15 decimal reductions which are equivalent to assuring a 10^{-6} SAL for contaminating populations per item of up to 10^9 micro-organisms each with a D_{121}-value of 1 min. The *USP* specification of 12*D* under the same assumption ensures an SAL of 10^{-6} for populations of up to 10^6 micro-organisms per item.

Thus, the pharmacopeial overkill specifications provide considerable degrees of assurance that SALs of 10^{-6} will be achieved. However, these high theoretical levels of overkill are contingent upon *D*-values in water being reflected by *D*-values in or on product. Most pharmaceutical products depress the thermal resistance of micro-organisms relative to their *D*-values in water, but this is not universally true. Some other materials (e.g., rubber) are known to increase the thermal resistance of micro-organisms (this may as likely be due to physical characteristics of heat transference as to biochemical protection). These product effects on thermal resistance can only be determined empirically, and are usually done in the laboratory using thermally resistant bacterial endospores, often spores of *Bacillus stearothermophilus*. Some previously unpublished guidance values on the effects of materials and pharmaceutical products on *D*-values of *B. stearothermophilus* relative to water are given in Table 1, and a detailed analysis has been published by Berger et al.[7] The use of *B. stearothermophilus* for this purpose and their frequent use as biological indicators (BIs) in bio-validation have contributed to a belief that steam sterilization must be defined in terms of being able to kill this micro-organism. It is not; spores of *B. stearothermophilus* are used with steam sterilization because of the convenience of their high resistance to steam sterilization and the unique and distinctive conditions required for their recovery and growth. Indeed, some major companies use *Clostridium sporogenes* or other species of *Bacillus* as reference or indicator organisms for this purpose.

The use of "overkill" specifications is not mandatory. In some instances, there may be pharmaceutical products which are unable to withstand the temperatures or energy inputs of overkill specifications. In these cases, specifications can be developed by calculating SALs of 10^{-6} from data characterizing the number of micro-organisms actually contaminating items before sterilization treatment, or from data characterizing the actual numbers and thermal resistances of the contaminating micro-organisms. The question is this—Is this exercise worth doing or would it be better and simpler to opt for aseptic manufacture of such heat-sensitive products?

Let us consider the number of micro-organisms contaminating pharmaceutical products prior to sterilization.

Table 1 *D*-values of spores of *B. stearothermophilus* on various substrates relative to water (*D*-value approximately 4 min)

Substrate	% relative to *D*-value in water (100%)
Stainless steel	60
Hydrophobic filter media	90
Silicone tubing	100
Rubber stoppers (various types)	85–150
Polycarbonate	120
Two pharmaceutical products pH 3.4–3.7	15–40
Pharmaceutical product pH 10.5–10.7	115

What are the highest and the lowest numbers which could be expected? For sterile parenteral products, the highest tolerable number of micro-organisms would be expected to be on the order of 10^2. This is because 10^3 or more per item is likely to begin to incur a risk of pyrogenicity. The lowest number which could be inferred from even an extensive number of zero counts would be one micro-organism.

Achievement of a 10^{-6} SAL from an initial bioburden of 10^2 would require eight log reductions. Applying these eight log reductions to an assumed worst case thermal resistance of D_{121}-value in water of 1 min gives a sterilization specification of 121°C for 8 min.

Achievement of a 10^{-6} SAL from an initial bioburden of 1 would require six log reductions. Applying these six log reductions to an assumed worst case thermal resistance of D_{121}-value in water of 1 min gives a sterilization specification of 121°C for 6 min.

The determination of thermal resistances is technically complex and requires special equipment (BIER Vessels). Since it is unlikely that *Bacillus* spp. can be excluded from any survey of microbiological contamination, it is reasonable to assume that spores with D_{121}-values on the order of 0.3 min will be isolated. Using this figure, SALs of 10^{-6} can be calculated at 121°C for 2.4 min for bioburdens of 10^2, and at 121°C for 1.8 min for bioburdens of one micro-organism per item.

The range of sterilization specifications calculable by these various approaches is summarized in Table 2. It is apparent that very brief sterilization specifications (on the order of 2–3 min holding time at 121°C) are obtainable when the microbiological contamination is completely characterized in terms of numbers and thermal resistances. In practice, such limits on hold times could be difficult to control precisely, are probably insignificant in terms of thermal lethality compared with heat-up and cool-down times, and could prove difficult to "sell" to regulators. Without complete thermal characterization of thermal resistances, specifications calculable by the "bioburden" approach are hardly significantly shorter than "overkill" specifications. Thus, it probably makes practical sense in most cases to choose only between overkill cycles for thermally resistant products and aseptic manufacture for heat-sensitive products.

Some products may be heat sensitive only above a threshold temperature; for those that can withstand temperatures in the range of 110–118°C but cannot withstand 121°C it is possible to apply the F_0 concept to the principles above and derive equivalent sterilization specifications to those given in Table 2. These specifications are summarized for 116°C in Table 3. As can be seen, if there is a requirement to sterilize at (say) 116°C, there are considerable time savings to be obtained by characterization of the contaminating micro-organisms.

Pharmaceutical Products and Materials for Aseptic Manufacture—Sterilizer Parameters

Sterilizer parameters are specific to combinations of product, presentation, and autoclave. They must be established empirically. Heat penetration studies done prior to the performance qualification phase of validation serve the purpose of determining the loading patterns, prevacuums, and temperature and pressure settings, etc. which ensure that the sterilization specification is delivered to the product and that it is delivered uniformly throughout the load.

For instance, a particular proposed loading pattern may never allow for uniform conditions (within specified limits) to be achieved throughout the load. In this case the pattern would have to be changed. Or, in a particular autoclave it may be necessary to set the temperature at 122°C for 121°C to be achieved within the load.

Air removal is particularly important in porous and equipment loads, but is usually of little importance in the sterilization of aqueous pharmaceutical products. Air removal can be important to the specification of new

Table 2 The range of sterilization specifications at 121°C calculable according to various approaches

	"Holding" time at 121°C (min)	
	Shortest possibility	**Longest possibility**
"Overkill" *PhEur*	15	Not specified
"Overkill" *USP*	12	Not specified
With bioburden[a] data only	6	8
With bioburden[a] and thermal resistance[b] data	1.8	2.4

[a] Assuming a "worst case" D_{121}-value of 1 min vs. bioburdens of one micro-organism (least) to 100 micro-organisms (most) per item.

[b] Calculated for a D_{121}-value of 0.3 min vs. bioburdens of one micro-organism (least) to 100 micro-organisms (most) per item.

Table 3 The range of sterilization specifications at 116°C calculable according to various approaches

	"Holding" time at 116°C (min)	
	Shortest possibility	**Longest possibility**
"Overkill" *PhEur*	48	Not specified
"Overkill" *USP*	38	Not specified
With bioburden[a] data only	19	26
With bioburden[a] and thermal resistance[b] data	5.7	7.6

[a] Assuming a "worst case" D_{121}-value of 1 min vs. bioburdens of one micro-organism (least) to 100 micro-organisms (most) per item. For equivalence a *Z*-value of 10 K has been used.
[b] Calculated for a D_{121}-value of 0.3 min vs. bioburdens of one micro-organism (least) to 100 micro-organisms (most) per item. For equivalence a *Z*-value of 10 K has been used.

autoclaves—those which are to be designated only for aqueous product sterilization have no need for the pumps and ancillary equipment required to pull deep vacuums.

The involvement of steam in the sterilization of different types of product is an important consideration in understanding and controlling autoclaves.

For aqueous products, steam is solely a means of raising the product to the specified sterilizing temperature; the steam does not come into contact with the contaminating micro-organisms. The transfer of heat energy (lethality) to the contaminating micro-organisms is from the product itself. To all intents and purposes any suitable form of energy source could be used to raise the temperature of the product. For instance, if ampules of aqueous products were to be sterilized in a hot air oven, the mechanisms of microbial inactivation would still be by coagulation of intracellular proteins. However, heat transfer from hot air is much slower than heat transfer from steam, which is why this is not seen as a practical process. Microwave irradiation could be an alternative means of sterilizing aqueous pharmaceutical products utilizing the same anti-microbial mechanisms as steam; certainly there is evidence that microwave killing patterns are mainly due to heat transfer with very little direct energy being absorbed from the microwaves.[8,9]

For porous and equipment loads, the steam comes into direct contact with the contaminating micro-organisms on the materials being sterilized and there is no intermediary in the transfer of heat. The energy content of steam is defined by its latent heat. If the steam is pure in the sense that it contains neither entrained gas nor moisture, an amount of energy defined by its latent heat at the pressure of the steam will be transferred to the micro-organisms by condensation on their surfaces.

There are many potential pitfalls in equipment and porous load sterilization, mainly concerned with air or other noncondensable gas. First, the purity of the steam is important; if it is carrying moisture, or noncondensable gas, it will not contain the same amount of energy as pure steam and its lethality will be less than that predicted for pure steam. Second, any residual air around the contaminating micro-organisms may insulate them from contact with the steam and thus reduce the amount of energy (lethality) transferred. In this type of sterilization, steam quality becomes very important and so also do the materials and manner in which the products are contained in steam-permeable wrapping or perforated trays, etc., within the autoclave, and the number and depth of evacuations of the autoclave prior to the temperature-hold phases. Thermal monitoring alone gives little information on the adequacy of the measures put in place to control these complex factors, and it is therefore generally thought essential that some empirical studies be done with BIs as part of process development to ensure that the thermal lethality being imparted by the steam is not being impeded. These development studies may be rolled into bio-validation.

Sterilization of Microbiological Media in the Laboratory

The various suppliers of microbiological media include recommendations for sterilization in their catalog under "Directions for Use," for instance, "sterilize by autoclaving at 121°C for 15–18 min." The question that must be asked is—What do these specifications mean?

Are they intended to apply within the media as are the sterilization specifications for pharmaceutical products and materials for aseptic manufacture? Or are they sterilizer parameters? There may be some indication in some of the older suppliers' manuals which expand their recommendations along the lines of "sterilize by autoclaving at 15 psi (121°C) for 15–18 min." Since pressures of 15 psi are not achievable within media, it is clear that the intention was that the recommendations be applied to sterilizer parameters.

In most cases, it is probably immaterial how these recommendations are interpreted. For media, "overcooking" is bad because of deleterious effects on growth-support characteristics, and "undercooking" is generally self-disclosing through evident contamination.

SIP Systems

Systems that are sterilized in-place are often immensely complex. The initial challenges to their sterilization are the removal of air and the elevation of the temperature of the pipework to prevent heat losses and condensation. As such, most work in the development of sterilization specifications for SIP systems is concerned with the heat-up phase. Appropriate questions are: Is the sterilization temperature achieved throughout the system? Where is the slowest location to achieve temperature? Where should the control probe be located?

Often vast amounts of thermal lethality calculated as F_0-values are delivered in these prehold stages of SIP. However, because these temperatures are being achieved in the presence of steam–air mixtures, it is not correct to assume that the biological lethality during the heat-up phase of SIP systems is equivalent to that achieved with pure steam.

The time for which the system must be held at temperature (the sterilization specification) is often relegated to a minor consideration compared with this earlier development work. Typically, it is decided arbitrarily to use , 121°C for 15, 20, or 30 min, with no real scientific basis.

Perhaps, a basis parallel to that of the pharmacopeial overkill specifications could be developed. For instance, if the actual maximum number of micro-organisms within an SIP system is assumed to be 10^{12} (since this would amount to a few grams of biomass it certainly should be maximal), then 18 log reductions would be required to ensure not more than one chance in a million of a survivor. An overkill cycle of 121°C for 20 min could be proposed by adding two log reductions as a safety factor and assuming each micro-organism to have a D_{121}-value of 1 min.

BIO-VALIDATION

The performance qualification (PQ) phase of validation follows the development of the sterilization specifications and of the sterilizer parameters which will deliver them. The purpose of PQ in steam sterilization of pharmaceutical products, equipment, laboratory media, and SIP systems is to confirm that the sterilization specification consistently achieves its intended purpose. The process is run using the parameters derived from process development on (usually) three separate occasions and tested for compliance with a variety of predetermined acceptance criteria. As a subset of PQ, the purpose of bio-validation is to confirm that the lethality expected from the process does not significantly deviate from what is expected. Bio-validation is a "test" of consistency. If the acceptance criteria are not achieved, there may be need for more process development.

In consideration of the extent, thoroughness, and history of the research evidence that micro-organisms are inactivated in a regular fashion in response to temperature and time, it is periodically suggested that bio-validation should not be necessary where there is evidence of adequate heat penetration. In practice, however, the expected lethality may not always be achieved. Most frequently, such deviations from ideality occur in equipment and porous load sterilization because of inadequate air removal. Where deviations from ideality occur for aqueous pharmaceutical products, they most likely arise from inadequate knowledge of how the product affects the thermal resistances of micro-organisms, but this is best determined in the laboratory at an earlier stage of process development, not at the bio-validation "milestone" later in the critical path of product introduction.

Acceptance criteria for bio-validation of steam sterilization processes are usually (but not invariably) defined along the following lines:

- *n* BIs will be placed in the load at locations defined in a drawing.
- Each BI will contain at least 10^6 viable spores of *B. stearothermophilus*.
- The load will be exposed to a defined autoclave treatment (the validation cycle).
- Bio-validation will be considered satisfactory if no viable spores are recovered from the BIs after *x* days of incubation at 55–60°C.

Because this approach is the common practice, there is a widely held belief within the pharmaceutical QA community that the ability to inactivate 10^6 spores of *B. stearothermophilus* is a synonym for achieving an SAL of 10^{-6}. It is not. It is true, however, that inactivation of 10^6 spores of *B. stearothermophilus* with the pharmacopeially approved minimum *D*-value of 1.5 min in 10–100 replicates guarantees achievement of better than 10^{-6} SALs for worst case bioburdens (Table 4). However, Table 4 also shows that the converse, i.e., failing to inactivate 10^6 spores of *B. stearothermophilus*, does not necessarily mean that a 10^{-6} SAL has not been achieved.

Another area of confusion is that the *USP* definition of an overkill specification—"a lethality input of 12*D*"—can be demonstrated directly in bio-validation. It should be

Table 4 Sterility assurance levels indicated by inactivation of 10^6 spores of *B. stearothermophilus*

	Spore *D* value			
	1.5 min	**2 min**	**3 min**	**4 min**
Bioburden of 10^2 micro-organisms per item each with *D*-values of 1 min	10^{-10}	10^{-14}	10^{-22}	10^{-30}
Bioburden of 10^2 micro-organisms per item each with *D*-values of 0.3 min	10^{-38}	10^{-51}	10^{-78}	10^{-104}
Bioburden of 1 micro-organism per item each with *D*-values of 1 min	10^{-12}	10^{-16}	10^{-24}	10^{-32}
Bioburden of 1 micro-organism per item each with *D*-values of 0.3 min	10^{-40}	10^{-53}	10^{-80}	10^{-106}

Inactivation of 10^6 spores in 10–100 replicates is assumed to be equivalent to 8 log inactivations.

understood that the maximum number of log inactivations of any bacterial population is technically limited to about 9 or 10 *D*-values. The maximum number of micro-organisms that can be handled as a BI is about 10^7–10^8, the sensitivity of recovery of micro-organisms is restricted to more than 10^{-2}. An indirect demonstration of 12 log inactivations of a micro-organism with a *D*-value of 1 min can be achieved by showing inactivation of 10–100 replicate BIs each carrying 10^6 spores with *D*-values of 1.5 min, or by inactivation of 10–100 replicate BIs each carrying 10^4 spores with *D*-values of 2 min. Direct demonstration of 12*D* is technically impossible.

In bio-validation, the spore of *B. stearothermophilus* is akin to an end-point analytical reagent. For instance, when litmus changes from blue to red at pH levels below 7, it shows only that the pH is not higher than 7. By killing all of 10–100 replicate BIs with 10^6 spores having *D*-value 1.5 min, all that is proven is that the thermal lethality delivered is not less than an F_0 of 12 min. The *PhEur* overkill sterilization specification of 121°C for 15 min should meet this requirement easily, and so should any other longer specification at 121°C, or any specification for longer times at lower temperatures taking into account of the F_0 concept.

Numbers and Locations of BIs for Bio-validation

It is usual for bio-validation to be done with an arbitrary number of BIs between 10 and 100. Both limits are based on practical considerations.

The lower number of BIs is defined in terms of ensuring that bio-validation addresses sufficient parts of the load for confidence that items in all parts of the autoclave are receiving the required lethality. Normal practice is to define this number in terms of placing at least as many BIs as the number of thermal probes used for thermal qualification. It is sensible to place one BI alongside each thermal probe in order to be able to relate thermal data to biological data. In addition to this, some BIs should be placed in other nonprobed locations in consideration of the possibility that the leads to the thermal probes may be acting as conduits for air removal or steam penetration, and thus provide falsely high levels of lethality.

More often than not the number of BIs used is about 20–30. Larger numbers up to 100 may be necessary to address very large autoclaves or in thermal mapping studies, but in validation there is little extra statistical confidence to be gained by doing so. In most microbiology QA laboratories, 20–30 BIs can be handled conveniently.

Periodically in the bio-validation of sterilization of porous or equipment "minimum" loads, it is not practical to locate 20 or 30 BIs. For instance, a minimum load may be one cartridge filter, one mop head, or one machine manifold. In such cases, it is important to avoid too much distortion of the statistics of bio-validation. At least five BIs are recommended no matter how difficult it may be to place them.

Choice of BIs

Spores of *B. stearothermophilus* are most commonly used for bio-validation of steam sterilization processes. This is not to say that it is mandatory to use *B. stearothermophilus* nor that it is used exclusively. Other micro-organisms, e.g., *sporogenes,* are used by some companies and accepted by the regulatory agencies. Use of *B. subtilis* spores with resistances to steam sterilization in the higher range of that found in natural bioburden has, in recent years, been criticized by European regulatory agencies.

The principles underlying the choice of micro-organism used as BIs are quite well known:

- The micro-organisms must have high resistance to the sterilization treatment which they are being used to validate. This does not mean that they must be the most resistant micro-organism known to man. *B. stearothermophilus* has D_{121}-values of 1–4 min according to conditions of culture and the substrate upon which they are mounted. This is higher than most spores of *Bacillus* spp., which tend to have D_{121}-values below 0.5 min.
- The micro-organisms must have stable resistances to the sterilization treatment which they are being used to validate. There are data from commercial suppliers of BIs to show that spores of *B. stearothermophilus* survive and retain stable resistances over long periods of crudely controlled storage.
- The micro-organisms must be easily culturable and preferably be easily identifiable in culture. Very few micro-organisms share with *B. stearothermophilus* the ability to grow in simple culture media at 55–60°C.

It is customary to use 10^6 (in practical terms 10^5–10^7) spores per BI. This number is based on custom, practice, and convenience rather than on science. Larger numbers than this are difficult to handle in culture and result in large errors in counting. Smaller numbers reduce the sensitivity of the test. Unfortunately, the widespread use of 10^6 spores for bio-validation has (as described before) led to a confusion between 10^{-6} SALs and 6 log reductions of *B. stearothermophilus*.

More complex decisions surround the choice of substrate within which spores are suspended or on which they are mounted for use as BIs.

The decision tree shown in Fig. 3 may be used to help choose the spore substrate used in bio-validation of aqueous pharmaceutical products. To use this decision tree, it is essential to have some knowledge of the effects of product on the resistance of spores; as mentioned before, this requires special equipment and experience.

In all circumstances water is the preferred substrate for bio-validation of aqueous pharmaceutical products. Where water would give deceptive results, it should not be used.

- If the D_{121}-value of *B. stearothermophilus* is higher in the product than it would be in water (i.e., the product makes the spores more resistant to steam sterilization), then bio-validation must be done with the spores suspended in product. Otherwise falsely favorable results may occur.
- If the D_{121}-value in product of *B. stearothermophilus* is equal to or less than its D_{121}-value in water, and the sterilization specification is based on overkill, then bio-validation must be done with spores suspended in water. However, if the sterilization specification has been "tailored" specifically to the resistance of micro-organisms in the product, then bio-validation must be done with the spores suspended in product. Otherwise falsely unfavorable results may occur.

Under no circumstances must spores be suspended in product if that product is sporicidal.

For other materials (for instance, equipment and supplies for aseptic manufacture) where the mechanisms of inactivation rely on direct contact between the steam and the item being sterilized and therefore where air removal is matter of importance, the choice of substrate for BIs generally lies between using commercially available paper spore strips and the material itself. The decision tree in Fig. 4 may be helpful. Regulatory pressure is currently toward use of inoculated product, but commercially available spore strips are more convenient. "Tailor-made" inoculated product requires substantial amounts of microbiological expertise. The decision tree in Fig. 4 may be helpful in selecting which approach is best in particular circumstances.

Use of commercially available BIs transfers much of the responsibility for assuring quality in manufacture to the supplier. Regardless of this, their quality must be controlled on receipt and prior to use in bio-validation. There is no reason why any microbiology laboratory should not verify the numbers of spores per commercial BI. On the other hand, the determination of resistance requires special equipment and expertise and is probably best accepted on the basis of the supplier's certification. If this is done, the user of the BIs is responsible for knowing what the certified measures of resistance mean, how they were determined (on the strips, in aqueous suspension, or in or on something else), and that they were determined correctly and in compliance with applicable standards and legislation.

Validation Cycle

Bio-validation is usually done against a sterilization specification which delivers less lethality than the lower limit of lethality allowed by the sterilization specification defined for the material being sterilized. It is clearly intellectually flawed to choose to validate something different to that which is ever to be used in practice. So what is the reasoning behind this practice?

Sterilization specifications in the "hold" period are presented in terms of temperature and time with upper and

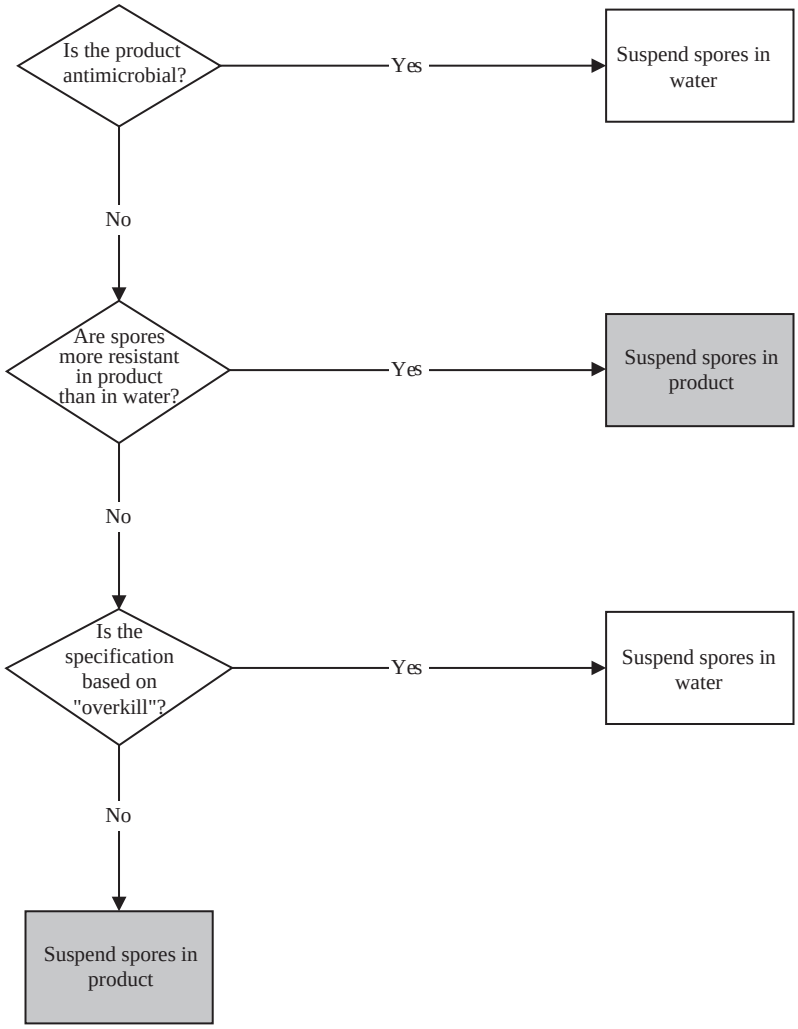

Fig. 3 Recommended substrates for BIs used in bio-validation of aqueous fluid loads.

lower tolerances set around them. The lower specification limits are critical to sterilization.

Time is generally easily controllable to quite high levels of accuracy and precision: A steam valve allows steam to enter the autoclave until the hold temperature is reached; the valve is then closed and the process is controlled by a timer which, at the end of the specified hold period, sends a control signal to activate the exhaust valves and cooling sequences. The hold time is usually specified in terms of whole minutes—well within the accuracy and precision of all but the most inappropriate of timers.

Temperature is less easy to control precisely. The temperature in the hold period in autoclaves is generally maintained by modulating valves which open to allow steam entry when the temperature (or pressure, because these valves are more often than not controlled through pressure transducers) begins to drop toward the critical lower limit of the specification. It is generally not possible to control an autoclave to run through a complete hold period at the lower limit of its temperature specification. However, even quite apparently trivial errors in temperature above or below the limit can make significant differences in the amount of lethality delivered. For instance, at a nominal temperature of 121°C, an error of 1 K can increase or decrease the amount of lethality by 25%.

Bio-validation cycles are therefore designed to ensure that no more lethality is delivered than that specified by the lower limits of lethality of the sterilization specification used in routine practice. Ideally this is done by reducing the time of the hold period, but sometimes, when quite short cycles are being used, it may also be necessary to reduce the temperature set point on the autoclave as well. The risk in all of this is that the bio-validation cycle is used as a justification for the release of sterilized items when

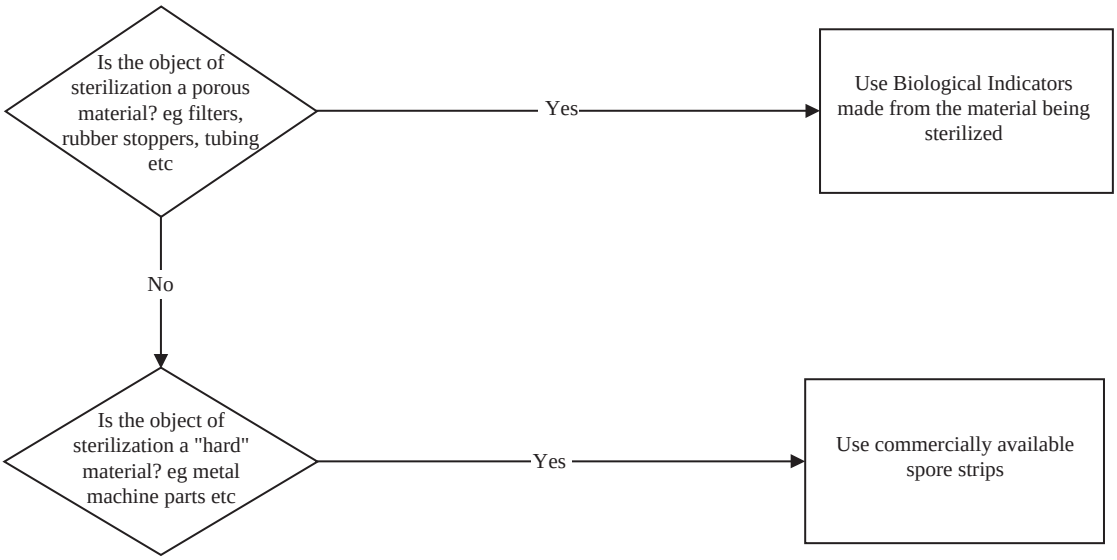

Fig. 4 Recommended substrates for BIs used in bio-validation of porous loads.

specifications are not complied with under atypical production conditions. This idea should not be entertained.

Acceptance Criteria

Bio-validation is a limit test, which at best produces quantal data. Each BI should be tested separately for survivors or absence of survivors. The acceptance criterion should be that there are no survivors.

Detection of survivors in all exposed BIs is clearly unacceptable; such a result would be obtainable if the BIs had never been near a sterilizer.

Data showing survival on some but not all of the BIs may be valuable in process development (particularly in the development of sterilization specifications and sterilizer parameters for porous and equipment loads), but would likely raise issues at regulatory inspection. The inference taken from having some survival could be that each item in the autoclave load is not being exposed to the same treatment.

For initial validation of a newly developed process, complete inactivation of all BIs should not be difficult to achieve.

The range of D_{121}-values acceptable to USP for spores of *B. stearothermophilus* allowed as BIs for use in steam sterilization is 1.5–3.0 min. An overkill sterilization or bio-validation specification delivering an F_0 of 15 min would deliver 10 log inactivations or, if there were 10^6 spores per BI, one chance in one million of finding a survivor on any one BI, one chance in one thousand of finding a survivor in 10 BIs, one chance in one hundred of finding a survivor in 100 BIs, and so on. In other words, there are pretty long odds against failing the acceptance criteria.

However, spores of *B. stearothermophilus* may have D_{121}-values of 3 min. In such a case there would be practically no chance of meeting the acceptance criteria of killing 10^6 spores with an F_0 of 15 min. What are the implications of this?

On the face of it, the implication is that this sterilization specification/sterilizer parameters combination is invalid. However, remember that this same sterilization specification/sterilizer parameter combination would have been valid if the BIs used had D_{121}-values of 1.5 min. In practical terms the pharmacopeial specification for thermal resistance in BIs has been set naively. Many companies purchasing spores of *B. stearothermophilus,* either for preparing BIs or as commercial strips, order against their own specifications which include upper D_{121}-value limits (in water) of around 2 or 2.5 min. The author of this paper has published recommendations[10] for determining bio-validation cycles appropriate to challenge numbers and D_{121}-values of the spores available, but in the long run it is far more convenient and easier to justify compliance to regulatory agencies when there is bio-validation to show that 10^6 spores have been inactivated in 10–100 replicates.

Requalification

Periodically it is wise to repeat bio-validation. Changes do occur in autoclaves and no change control procedure, no matter how rigorously implemented, is infallible. The

purpose of requalification is to determine if any unforeseen change has arisen to affect the sterility assurance provided to the items being sterilized.

It is important for requalification that the numbers, resistances, and substrates for the BIs are closely similar to those used in the initial validation. For the same reasons as resistance variation within BIs as discussed before, it is possible if these factors are not well controlled to emerge from requalification with either a false confidence in the security of the process (use of BIs which are less resistant than those used in initial validation), or with the incorrect opinion that the process has failed (use of BIs which are more resistant than those used in initial validation).

Biological requalification is usually done following significant process changes or on an annual frequency. The establishment of a frequency should, in principle, be based on business risk; in fact, however, with well-designed and controlled autoclaves, the risk to the business of extending the interval beyond 1 yr is probably more one of incurring regulatory criticism at inspection than of releasing nonsterile products to market.

Bio-validation of Laboratory Autoclaves Used for Sterilizing Microbiological Media

It is not difficult to argue that the effectiveness of sterilizing microbiological media is self-disclosing and should not therefore merit bio-validation. The pertinence of bio-validation to the qualification of laboratory autoclaves is more to do with having confidence in the sterility of media before or after it is used in the laboratory (and risk false positive results if the media is not sterile), or take it into (say) aseptic manufacturing areas for environmental monitoring (and if it is nonsterile contaminate areas and products which may otherwise have been secure). Many regulatory bodies see bio-validation of laboratory autoclaves as mandatory.

Bio-validation of Steam-in-Place (SIP) Systems

SIP systems range from very small systems (say, a mixing tank) where all parts may reach temperature within 2 or 3 min, to absolutely massive arrangements of vessels, valves, and pipework in which the expulsion of air, the drainage of condensate, and the attainment of the sterilization specification temperature at the "slowest point" can take 20 or 30 min.

Bio-validation is essential. The placement of BIs is largely a matter of judgment. Certainly the "slowest point" to reach the sterilization specification temperature must be challenged. Certainly vent filters and low points where condensate could accumulate must be challenged. Thereafter, it is a regulatory expectation that there should be sufficient BIs placed to give coverage to the whole system, which in effect may mean placing BIs in locations which, due to the heat-up time of the system, have been exposed to the sterilization specification temperature for two, three, or four times longer than the "slowest point." Undue confidence should not be taken from favorable results from these locations.

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